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DOI: <https://doi.org/10.1159/000329957>

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ZORA URL: <https://doi.org/10.5167/uzh-59852>

Journal Article

Published Version



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Originally published at:

Felder, K M; Hoelzle, K; Ritzmann, M; Kilchling, T; Schiele, D; Heinritzi, K; Groebel, K; Hoelzle, Ludwig E (2011). Hemotrophic mycoplasmas induce programmed cell death in red blood cells. *Cellular Physiology and Biochemistry*, 27(5):557-564.

DOI: <https://doi.org/10.1159/000329957>

Hemotrophic Mycoplasmas Induce Programmed Cell Death in Red Blood Cells

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Key Words

Eryptosis • Hemotrophic mycoplasmas • Hemolytic anemia • Infection

Abstract

Hemotrophic mycoplasmas (HM) are uncultivable bacteria found on and in the red blood cells (RBCs). The main clinical sign of HM infections is the hemolytic anemia. However, anemia-inducing pathogenesis has not been totally clarified. In this work we used the splenectomized pig as animal model and *Mycoplasma suis* as a representative for hemotrophic mycoplasmas to study anemia pathogenesis. Eryptosis, i.e. programmed cell death of RBCs, is characterized by cell shrinkage, microvesiculation and phosphatidylserine (PS) exposure on the outer membrane. The eryptosis occurrence and its influence on anemia pathogenesis was observed over the time-course of *M. suis* infections in pigs using 3 *M. suis* isolates of differing virulence. All 3 isolates induced eryptosis, but with different characteristics. The occurrence of eryptosis could as well be confirmed *in vitro*: serum and plasma of an acutely ill pig induced PS exposure on erythrocytes drawn from healthy pigs. Since *M. suis* is able to induce eryptotic processes it is concluded that eryptosis is one anemia-inducing

factor during *M. suis* infections and, therefore, plays a significant role in the pathogenesis of infectious anemia due to HM infection.

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Introduction

Hemotrophic mycoplasmas (HM) are a group of uncultivable bacteria that are closely associated with red blood cells of several mammals. Currently, cases of humans infected with HM were reported which led to growing interest of the mycoplasma community in this specific research field [1-3]. Affected patients suffered from fever, splenomegaly and autoimmune hemolytic anemia (AIHA). Since 45 % of warm AIHA are classified as idiopathic, i.e. no underlying disease is recognized, we hypothesize that HM could be involved in the pathogenesis [4]. In China, the studies revealed *Mycoplasma suis* as causative agent of human HM infections [5]. This bacterium mainly infects pigs and is responsible for a disease referred to as infectious anemia of pigs (IAP). Clinical signs caused by high *M. suis* loads are raised body temperature, severe anemia, labored breathing due to insufficient oxygen transport and hypoglycemic shock which sometimes is lethal. Tetracycline therapy cures from

clinical symptoms in the majority of cases. However, *M. suis* is not completely eradicated from the host. The following chronic phase of *M. suis* infection is characterized by milder anemia, higher susceptibility to other infections and occurrence of acrocyanosis due to cold agglutinins [6, 7]. This chronic phase is interrupted by *M. suis* growth peaks accompanied by the aforementioned clinical signs. These “clinical attacks” get milder each time and the pigs usually survive without antibiotic therapy after the third attack [8].

To date two main pathogenesis mechanisms are known to be responsible for the development of the severe and often fatal hemolytic anemia during *M. suis* infections: (I) direct interaction between *M. suis* and the red blood cell, including invasion which leads to cell damage and subsequent lysis [9, 10]; and (II) upregulation of autoreactive antibodies targeting either the host actin or so far unspecified glycoproteins on the red blood cell surface [6, 11]. Recently, electronmicroscopic investigations of blood from experimentally *M. suis* infected pigs [9] revealed red blood cells exposing tiny vesicles on their surface and attributed them to erythrocytes undergoing suicidal cell death. It is known that red blood cells that are under stress due to infection or other reasons like exposition to oxidative environments in the lung, osmotic changes or mechanic squeeze can undergo programmed cell death [12–15]. The process is referred to as eryptosis and characterized by cell shrinkage, membrane blebbing, activation of proteases and phosphatidyl-serine exposure on the outer membrane leading to recognition by macrophages and, therefore, phagocytosis [16]. Interestingly, eryptosis was also described to play a role in the pathogenesis of malaria. This is a further well-known blood associated disease in which parasites invade red blood cells leading to clinical signs that include anemia and undulating fever [14].

In this study we investigated the occurrence of eryptosis in the course of experimentally induced infections with three *M. suis* isolates differing in virulence. Our investigations focused on the analysis and the potential impact of eryptosis on the severity of the anemia observed during IAP.

Materials and Methods

Pigs

A splenectomized pig model was used to perform the experimental infections [17]. Weaned piglets (female, German Landrace, 5 weeks old) were housed in a pen of 20 m² in groups of 4 animals. The negative *M. suis* status was confirmed by

quantitative *M. suis* PCR and *M. suis* immunoblot as described previously [18, 19]. At the age of 7 weeks pigs were infected by intramuscular injection of 1 ml of *M. suis*-containing blood (10⁶ to 10⁷ bacterial cells per ml) and bled twice a week for sampling. From the day of infection clinical signs, feeding behavior and body temperature of each individual pig were controlled daily. To evaluate the clinical status a previously described scoring system was used [20]. Briefly, a score of 1 was given each for the occurrence of fever (body temperature > 40°C), lethargy, reduced food uptake, and pale/ear necrosis. For each animal, these scores were added and acutely ill pigs with a clinical score of 4 were treated with tetracycline (40 mg/kg body weight) and glucose (35 g glucose / l drinking water). Animal experiments were conducted with the approval of the Veterinary Office of Zurich, Switzerland, under the registration number 55/2007 with legal prescriptions.

M. suis isolates

Three different *M. suis* isolates were used. They were classified according to their virulence, as low, moderate and high. The low virulent isolate (lv, referred to as 146/5 [GenBank:FN391021.1]) is not invasive, induces weak clinical signs earliest 14 days post infectionem (dpi), and shows minimal mortality. Antibiotic treatment leads to significant improvement of clinical signs. The moderately virulent isolate (mv, referred to as 3804 [GenBank:FN984917.1];[21]) is not invasive with the first clinical signs being observed from 10 dpi. Antibiotic treatment leads to an attenuation of clinical signs; mortality after infection with 3804 is high. The highly virulent isolate (hv, referred to as KI_3806 [RefSeq:NC_015153];) is invasive and antibiotic treatment is ineffective. Experimentally infected pigs die within 10 dpi (nearly 100% mortality). These animals were euthanized and exsanguinated to relieve their suffering.

Quantitation of M. suis

M. suis loads were quantified as previously described [18]. Briefly, DNA was extracted from 200 µl EDTA-anticoagulated blood using the blood and tissue kit (Sigma, Buchs, Switzerland) according to the manufacturer's recommendations, and analyzed by quantitative PCR targeting the *gl* adhesin gene of *M. suis*. A standard curve was measured in parallel to calculate *M. suis* genome equivalents.

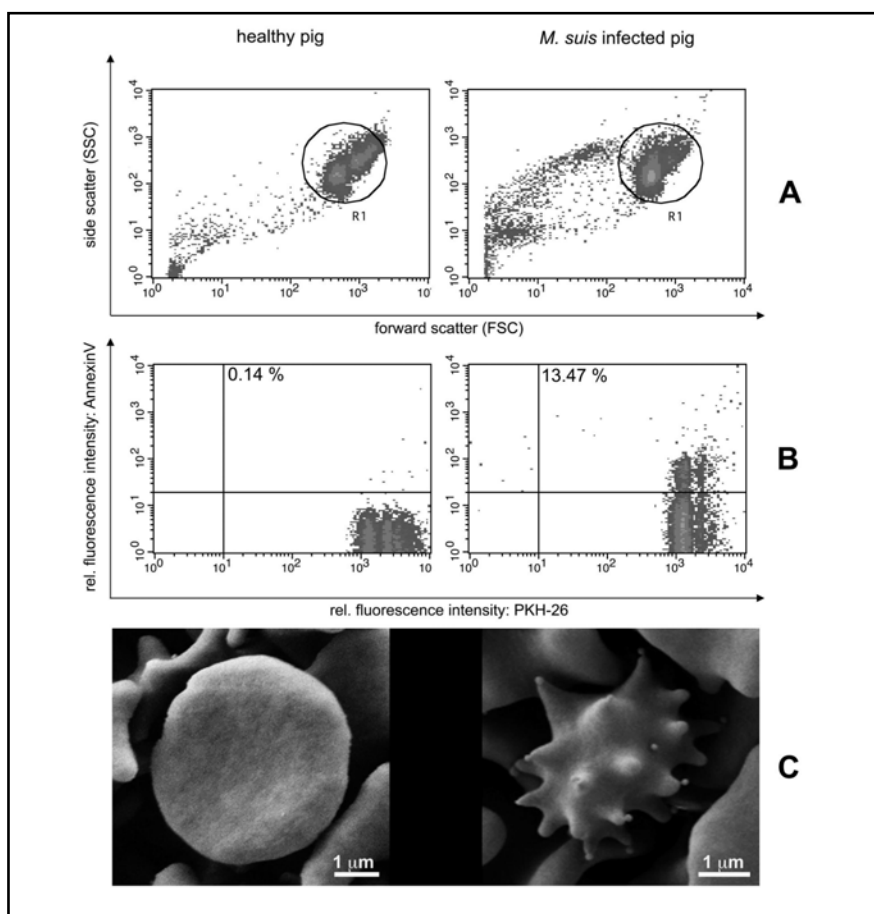
Hematologic parameters

White blood cell count including differential blood cell analysis, red blood cell count, hemoglobin concentration and hematocrit values were determined using a 3-DIFF Analyzer (ABX Micros CRP, AxonLab, Baden, Switzerland) according to the manufacturer's recommendations. EDTA anticoagulated blood was tested within 1h of withdrawal.

Staining of red blood cells

To measure eryptotic cells, red blood cells were purified from EDTA blood by 3 washes with 1xPBS (400 x g, 5 min, 20°C). Approximately 10⁷ cells were stained with PKH-26 (Sigma), a membrane cell linker dye to stain cells with intact cell membranes. To detect cells showing phosphatidyl-serine exposure, the erythrocytes were counterstained with 100 µl

Fig. 1. Illustration of measuring eryptotic cells infected with *M. suis*. A: Density plots of the erythrocyte population. 10000 cells in gate R1 were considered for quantitations. B: Density plots showing the relative fluorescence intensities of cells in gate R1. The eryptotic and, therefore, double-stained cells are found in the upper right quadrant. Numbers indicate percentage of double-stained cells in gate R1. The example represents numbers found in erythrocytes infected with the highly virulent strain. C: Scanning electron micrographs of a discoid, normal red blood cell (left) and a spheroid eryptotic cell with microvesiculation (right) are shown. Scale bars indicate 1 μ m.



AnnexinV-Fluos working solution (Roche) for 15 min. The reaction was stopped by addition of 500 μ l HEPES Buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl_2). Cells were immediately analyzed with a fluorescence activated cell-sorting device (FACSCalibur, BD Biosciences, Switzerland). 10,000 red blood cells were recorded; double-stained cells considered as to be eryptotic were quantified. A cut-off value was set at the mean plus 3 times standard deviation of the negative controls (n=10).

To set parameters on the FACSCalibur, eryptosis was induced in freshly drawn erythrocytes by means of incubation with 0.5 μ M Ionomycin (Sigma) and 2.5 mM CaCl_2 for 10 min at 37 $^\circ\text{C}$. The cells then were stained as described above. Accordingly, freshly drawn red blood cells from healthy pigs were stained. AnnexinV-Fluos and PKH-26 fluorescence intensities were recorded on Fluorescence channel FL-1 and FL-2, respectively. Electronic compensation was adopted (FL-1 – 9 % FL-2; FL-2 – 14.5 % FL-1) to exclude overlapping of the emission spectra.

In vitro induction of eryptosis

Cells were stained with PKH-26 as described above. About 10^6 stained cells were incubated overnight at 37 $^\circ\text{C}$ with 100 μ l of serum or plasma taken 14 dpi from a pig infected with the mv *M. suis*-isolate. The serum and plasma samples were stored in aliquots at -20 $^\circ\text{C}$ until use. As controls serum and plasma obtained from the same pig before infection with *M. suis* were used.

Microscopy

Scanning electron microscopy (SEM) was performed as previously described [9]. Briefly, red blood cells were fixed in 2.5% glutaraldehyde immediately after withdrawal and stored at 4 $^\circ\text{C}$ until use. Cells were settled on carbon-coated coverslips, dehydrated in increasing concentrations of acetone and dried. They were sputter-coated by means of 10 nm platinum and examined with a Zeiss Supra 50 VP scanning electron microscope.

Statistical analysis

To test whether 2 groups differed significantly in eryptosis properties, *P*-values were calculated using the Student's *t*-test. Two groups were considered significantly different from each other if *P* < 0.05.

Results

Detection of eryptosis in M. suis infected pigs

Eryptotic cells are mainly characterized by the breakdown of membrane asymmetry leading to exposure of phosphatidyl-serine (PS)-residues. To demonstrate and quantify eryptotic cells in *M. suis* infected pigs specific staining of exposed PS- residues with AnnexinV-Fluos was performed. Since eryptotic

<i>M. suis</i> isolate	animal experiment no	dpi ^a	% dsc ^b	bacterial load per ml blood	clinical score	hematocrit (%)	status
highly virulent	1	6	0.48	1.20 x 10 ⁶	0	34.2	died
		9	n.d. ^c	4.21 x 10 ¹⁰	4	26.1	
	2	3	0.41	5.32 x 10 ³	0	36.3	euthanized
		8	18.61	1.61 x 10 ¹¹	4	25.5	
	3	3	0.22	1.49 x 10 ¹	1	26.9	euthanized
		8	14.33	6.62 x 10 ⁸	3	29.4	
		10	0.46	1.23 x 10 ⁷	4	10.3	
	4	3	1.37	2.42 x 10 ³	0	36.2	euthanized
		8	13.04	2.38 x 10 ⁹	2	36.1	
		9	19.35	2.03 x 10 ¹⁰	3	33.2	
		10	0.29	3.62 x 10 ¹⁰	4	25.4	
moderately virulent	5	2	1.37	2.42 x 10 ³	0	36.2	euthanized
		7	4.54	1.10 x 10 ³	0	39.4	
		10	0.91	7.66 x 10 ⁵	0	38.6	
		12	1.61	1.40 x 10 ¹¹	3	36.1	
		14	1.99	7.22 x 10 ¹¹	4	20.8	
		16	1.39	6.10 x 10 ⁷	4	14.4	
	6	2	0.14	2.43 x 10 ³	1	26.9	euthanized
		7	6.29	2.38 x 10 ⁹	1	36.4	
		9	5.28	2.03 x 10 ¹⁰	3	32.7	
		10	0.22	3.62 x 10 ¹⁰	4	24.9	
	7	2	0.15	2.47 x 10 ⁵	0	35.2	euthanized
		7	6.55	7.65 x 10 ³	0	36.7	
		10	1.84	3.65 x 10 ⁵	0	35.2	
		12	15.6	8.42 x 10 ⁸	4	28.6	
	8	0	0.15	b.d.	0	36.7	euthanized
		6	0.71	5.28 x 10 ¹	0	37.4	
		12	n.d.	5.58 x 10 ⁶	4	27.0	euthanized
	9	18	0.33	b.d. ^d	0	38.0	
		21	1.40	b.d.	0	36.4	survived
		27	3.09	6.25 x 10 ³	0	34.2	
		32	1.70	1.06 x 10 ³	0	36.4	
		35	1.91	2.60 x 10 ¹	0	35.1	
		41	0.05	b.d.	0	33.3	
low virulent	10	7	0.57	b.d.	0	38.4	survived
		11	1.20	b.d.	0	37.7	
		14	n.d.	b.d.	0	38.8	
		18	1.00	b.d.	0	32.0	
		21	0.76	b.d.	0	35.8	
		7	0.78	b.d.	0	39.6	survived
	11	11	0.54	b.d.	0	38.8	
		14	n.d.	b.d.	0	42.2	
		18	1.36	b.d.	0	35.6	
		21	1.40	b.d.	0	37.7	
	12	0	0.15	b.d.	0	36.7	survived
		4	n.d.	b.d.	0	39.0	
		7	1.73	b.d.	0	38.4	
		11	0.37	b.d.	0	34.9	
		14	n.d.	b.d.	0	33.4	survived

Table 1. Overview of the obtained data. 3 groups of pigs (n=4) were infected with 3 different *M. suis* isolates. Eryptosis activity was compared to the severity of the anemia (hematocrit) and the bacterial loads. ^adays post infectionem. ^bpercent of double-stained red blood cells. ^cnot done. ^dbelow detection limit of the quantitative PCR.

cells could be differentiated from necrotic cells by their maintenance of the plasma membrane integrity a

membrane staining with PKH-26 was performed to tag cells with intact cell membranes.

Red blood cells from *M. suis* negative pigs treated with Ionomycin and CaCl_2 (induction of PS-exposure) were used as positive controls. Untreated erythrocytes were used as negative controls. An eryptosis cut-off was determined at 1.15 %.

In blood samples taken from *M. suis* infected animals during acute IAP the amounts of eryptotic red blood cells (positive for both, AnnexinV-Fluos and PKH-26) were significantly higher ($P < 0.05$) when compared to blood samples from healthy pigs (Fig. 1). The occurrence of eryptotic cells in the blood of *M. suis* infected pigs could be confirmed by scanning electron microscopic investigations due to their characteristic morphological changes i.e. cell shrinkage, change in shape from discoid to spheroid, and microvesiculation (Fig. 1).

Quantitation of eryptosis during the course of M. suis infections

To evaluate the impact of eryptosis on the severity of anemia in *M. suis* infections, 3 groups of pigs (n=4) were infected with differently virulent isolates of *M. suis*, i.e. highly virulent (hv), moderately virulent (mv) and low virulent (lv). The relation between clinical signs (i.e. clinical scores), anemia associated parameters (i.e. hematocrit values), *M. suis* loads, and the ratio of eryptosis (i.e. AnnexinV-Fluos and PKH-26 double-stained red blood cell count) was evaluated (Table 1).

In pigs infected with the hv *M. suis* isolate the disease progressed extremely fast and the clinical outcome was fatal with clinical scores = 4 by 10 dpi at the latest and high *M. suis* loads (up to 10^{11} /ml blood). The severity of anemia and the bacterial loads correlated with the amount of double stained red blood cells in these animals (Table 1, Fig. 2). The proportion of eryptotic cells in the blood at the peak of anemia amounted up to 19.35 %. Shortly before euthanasia, red blood cells often were no longer stainable with PKH-26. At this time, membrane damage was too severe and intravascular lysis evident. Therefore, the values of eryptotic cells fell to a minimum, with no double-stained cells detected anymore.

The pigs infected with the mv *M. suis* isolate demonstrated eryptosis activity from day 7 pi. At this point the proportion of eryptotic cells in the blood amounted to a maximum of 6.55 % with a clinical score of 0 and an *M. suis* load of 10^3 cells per ml blood. No direct correlation between eryptotic cells and *M. suis* loads was observed. Three animals had to be euthanized at dpi 12 on the latest. In blood samples of the remaining pig weak eryptosis could be detected. In parallel, the bacterial loads increased

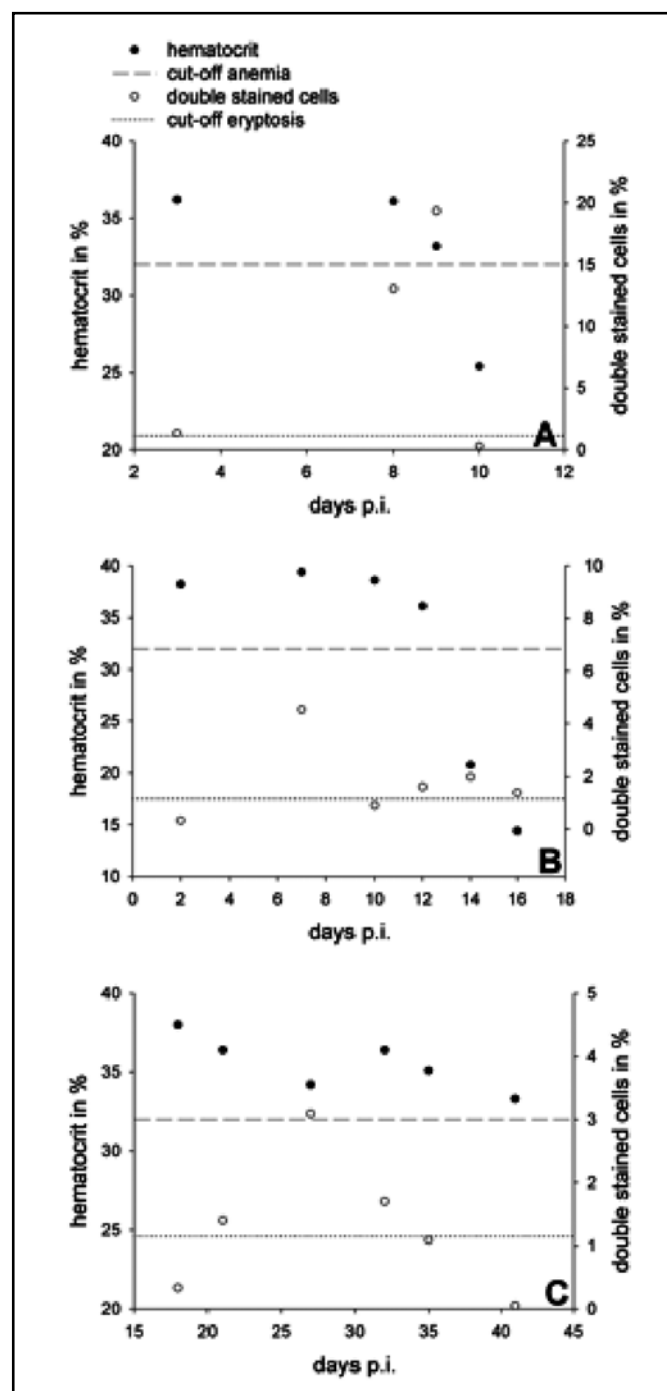


Fig. 2. Correlation of double-stained red blood cells with hematocrit and, hence, the severity of anemia. The cut-off for eryptosis occurrence, i.e. 1.15 % double-stained cells and for anemia, i.e. hematocrit <32 % [33] are indicated. A: Infection with the highly virulent isolate. The severity of anemia negatively correlates with the amount of double-stained red blood cells. At day 10 p.i., the cells were no longer stained with PKH-26 due to severe damage and intravascular lysis. B: Infection with the moderately virulent *M. suis* isolate. At the beginning of the infection eryptosis is upregulated. Later on anemia progresses without appreciable eryptotic activity. C: Infection with the low virulent *M. suis* isolate. Eryptosis is observed from day 21 pi. Hematocrit levels are constant.

Treatment	% double-stained red blood cells ^a
Untreated	0.53 ± 0.26
Serum A (<i>M. suis</i> positive)	2.31 ± 0.35*
Serum C (<i>M. suis</i> negative)	1.63 ± 0.50
Plasma A (<i>M. suis</i> positive)	5.14 ± 3.54*
Plasma C (<i>M. suis</i> negative)	2.97 ± 2.14

Table 2. Double-stained red blood cells after incubation with serum and plasma. ^aValues are means ± standard deviations of three independently performed experiments (5 samples each) for AnnexinV-FLUOS and PKH-26 double-stained cells as a percentage of 10000 examined red blood cells from healthy pigs incubated overnight with serum and plasma samples from an acutely ill *M. suis* infected pig (serum A, plasma A) or controls (serum C, plasma C), respectively. Accordingly, cells were stained prior to incubation (untreated). *Significantly different ($P < 0.05$) from value for treatment with controls as calculated by Student's *t* test.

up to 7.22×10^{11} and anemia progressed. After tetracycline treatment the bacterial loads decreased by day 16 pi but the animal developed a severe anemia (clinical score = 4) and had to be euthanized.

In animals infected with the lv *M. suis* isolate eryptotic double-stained cells could be detected as well. The amount of double-stained cells remained below 3.5 %. If detectable, the *M. suis* blood loads were significantly lower than in the hv and mv pig groups (maximum value 6.25×10^3 /ml blood) and correlated directly with the percentage of eryptotic double-stained red blood cells ($R^2 = 0.98$). The animals showed no clinical signs during this time (clinical scores = 0), and the hematocrit never decreased below 32 %.

In vitro induction of eryptosis by *M. suis*

To confirm the presence of eryptosis inducing factors in *M. suis* infected pigs, red blood cells taken from healthy *M. suis* negative pigs ($n = 3$) were incubated with serum or plasma from experimentally *M. suis* infected pigs at acute IAP. Control experiments were performed using serum or plasma from the same pigs prior to *M. suis* infection. The *M. suis* loads of serum and plasma were 9.74×10^8 per ml and 6.08×10^{11} per ml, respectively.

Both the incubation with *M. suis* positive serum/plasma and *M. suis* negative serum/plasma led to an increase of double-stained cells when compared to untreated red blood cells (Table 2). However, the *M. suis* treated red blood cells (plasma and serum) showed significantly higher eryptosis rates than the controls in all experiments ($P < 0.05$; Table 2).

Discussion

Under stress erythrocytes undergo conformational changes, which are well known features of apoptosis in nucleated cells, the process in this specific case being referred to as eryptosis [12, 13, 15]. Several stimuli and diseases trigger eryptosis, including energy depletion, oxidative stress, osmotic shock, cyclosporine, peptidoglycans, prostaglandin E_2 , sepsis, malaria and glucose-6-dehydrogenase-deficiency [22-28].

In acute *M. suis* infections the main clinical sign is severe hemolytic anemia. Upregulation of autoreactive antibodies targeting actin during disease states occurs with high *M. suis* loads [11]. These antibodies only bind actin on damaged cells because their actin becomes accessible. The close association of *M. suis* cells with the erythrocytes leads to remarkable alterations in the cytoskeleton and the cell membrane [29]. Erythrocyte membrane alterations could be induced by ion channel formation. Such a mechanism is known from *Plasmodium falciparum*, another invasive erythrocyte-associated pathogen. The channels are used to uptake nutrients, Na^+ and Ca^{2+} ions, and for disposal of waste products [22, 30]. This leads to upregulation of eryptosis, removal of infected cells and, therefore, removal of parasites from the body. Hence, cell suicide offers a defense mechanism against malaria [14]. For other hemotrophic parasites, for instance HM, nothing is known about erythrocyte suicide processes. By analogy to malaria, we hypothesize that *M. suis* could induce eryptosis as a side effect of membrane alterations induced during invasion [9].

In this study we have described for the first time the occurrence of eryptosis in HM infections using the established splenectomized pig model for *M. suis* infections [17]. Generally, the frequency of eryptosis depended on the different *M. suis* isolates used for infection. In those pigs infected with the highly virulent isolate eryptosis was upregulated, but failed to control the infection. Erythrocytes were lysed intravascularly by other mechanisms such as autoreactive antibodies targeting actin, or directly by *M. suis* [11]. Pigs infected with the low virulent *M. suis* isolate never showed any clinical signs. Double-stained red blood cells were present. A measurable load of *M. suis* cells was detected (6.25×10^3 /ml blood) during this time. Here, eryptosis successfully prevented a fatal infection-course as seen by only a slight decrease in the hematocrit. In pigs infected with the moderate *M. suis* isolate eryptosis was detectable early (2-7 dpi), but only a low amount of double-stained cells

was found in later blood samples when the protective mechanism failed, and infection got fatal since *M. suis* was able to grow. Therefore, early anemia is due to elimination of infected red blood cells. Potential removal of red blood cells by phagocytosis in early *M. suis* induced anemia was noted earlier, but without any experimental evidence [6]. We can confirm the influence of programmed cell death and, therefore, of the involvement of phagocytosis in the anemia pathogenesis.

Eryptosis was inducible *in vitro* in erythrocytes from healthy animals that had been incubated overnight with serum and plasma samples taken on from an acutely ill pig infected with the mv isolate. This implies that *M. suis*, soluble *Mycoplasma* substances or stress signals present in the blood of diseased animals are responsible for induction of the eryptosis. It is noteworthy that a higher rate of eryptosis was reported in human sepsis patients [24]. In that study erythrocytes of healthy volunteers were incubated with plasma from sepsis patients, which triggered phosphatidyl-serine exposure and, therefore, eryptosis. The effect was attributed to the accumulation of sphingomyelinase-induced intraerythrocytic ceramide [31]. The reason for sepsis in these patients was diverse; a bacterial causative agent was identified in 8 patients, and in one case *M. pneumoniae* was identified. Additionally, active substances produced by the pathogen can elicit induction of eryptosis. As an example, *E. coli* hemolysin induced structural changes in sheep

erythrocytes i.e. they became spherical and formed 10-20 small vesicles on their surface [32]. This only occurred in medium containing calcium.

It is not clear what kind of signal is used for triggering suicidal red blood cell death in *M. suis* infections. *M. suis* cells were present in both inducing samples. Generally, plasma induced a higher percentage of double-stained red blood cells than serum. However, plasma samples contained higher loads of *M. suis* cells.

We evaluated the occurrence of programmed cell death of erythrocytes in pigs infected with the hemotropic mycoplasma *M. suis* thus providing further evidence in elucidating the pathogenesis of IAP. Interestingly, a correlation of eryptosis with the severity of the anemia was observed. This is important in terms of developing of novel therapy and prophylaxis strategies against the HM induced anemia. With this study we contribute a further puzzle-piece towards the elucidation of pathogenesis mechanisms of HM.

Acknowledgements

This work was supported by the Forschungskredit of the University of Zurich, Switzerland (55130101 to K.M. Felder) and by the Swiss National Science Foundation (310030-127189 to L.E. Hoelzle).

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